A Comparative Study of the Infrared Difference Spectra for Octopus and Bovine Rhodopsins and Their Bathorhodopsin Photointermediates†

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ABSTRACT: Fourier-transform infrared difference spectroscopy has been used to detect the vibrational modes in the chromophore and protein that change in position and intensity between octopus rhodopsin and its photoproducts formed at low temperature (85 K), bathorhodopsin and isorhodopsin. The infrared difference spectra between octopus rhodopsin and octopus bathorhodopsin, octopus bathorhodopsin and octopus isorhodopsin, and octopus isorhodopsin and octopus rhodopsin are compared to analogous difference spectra for the well-studied bovine pigments, in order to understand the similarities in pigment structure and photochemical processes between the vertebrate and invertebrate systems. The structure-sensitive fingerprint region of the infrared spectra for octopus bathorhodopsin shows strong similarities to spectra of both *all-trans*-retinal and bovine bathorhodopsin, thus confirming chemical extraction data that suggest that octopus bathorhodopsin contains an *all-trans*-retinal chromophore. In contrast, we find dramatic differences in the hydrogen out-of-plane modes of the two bathorhodopsins, and in the fingerprint lines of the rhodopsins and isorhodopsins for the two pigments. These observations suggest that while the primary effect of light in the octopus rhodopsin system, as in the bovine rhodopsin system, is 11-cis/11-trans isomerization, the protein–chromophore interactions for the two systems are quite different. Finally, striking similarities and differences in infrared lines attributable to changes in amino acid residues in the opsin are found between the two pigment systems. They suggest that no carboxylic acid or tyrosine residues are affected in the initial changes of light–energy transduction in octopus rhodopsin. Comparing the amino acid sequences for octopus and bovine pigments also allows us to suggest that the carboxylic acid residues altered in the bovine transitions are Glu-122 and/or Glu-134.

In both vertebrates and invertebrates, the process of vision is initiated by absorption of light by rhodopsin, a retinal-containing protein found in the photoreceptor cells of the eye. The absorption of light by rhodopsin results in the formation of a red-shifted photointermediate known as bathorhodopsin. Bathorhodopsin then decays thermally via a series of intermediates, eventially triggering visual excitation through a transduction cascade (Stryer, 1986; Hurley, 1987). The sequence of intermediates that result from the absorption of light by rhodopsin has been studied extensively for both vertebrate and invertebrate systems [see, e.g., Yoshizawa (1972), Ottolenghi (1980), and Tsuda (1987)].

The rhodopsin-to-bathorhodopsin transition is of central importance in understanding the photophysics of vision since it involves the conversion of light to the chemical free energy responsible for visual transduction (Honig et al., 1979; Cooper, 1979; Cooper et al., 1986; Schick et al., 1987). To date, most of our understanding of this process has been obtained from studies of bovine rhodopsin. In particular, resonance Raman (Callender et al., 1976; Mathies et al., 1977; Eyring et al., 1982; Palings et al., 1987) and infrared (Bagley et al., 1985, 1987; Rothschild et al., 1983; Siebert et al., 1983) studies have provided a wealth of information concerning the structure of the retinal chromophore in the various intermediates of the photosequence. In addition, infrared studies have been used to study changes that the opsin experiences in the early stages of visual transduction (Siebert et al., 1983; Bagley et al., 1985). In contrast, relatively little is known about the invertebrate rhodopsins and their photointermediates. Squid and octopus rhodopsins are perhaps the best characterized and most readily available of the invertebrate pigments [see Tsuda (1987) for a recent review].

There are significant similarities in the vertebrate and invertebrate rhodopsins as exemplified by comparison of bovine rhodopsin and octopus rhodopsin and their respective photochemistries. Chemical extraction studies have shown that the chromophore in octopus rhodopsin, like that found in bovine rhodopsin, is an 11-cis isomer of retinal (Hubbard & St. George, 1958; Pande et al., 1987; and the data presented herein). Illumination of both bovine rhodopsin and octopus rhodopsin at physiological temperatures results in the formation of bathorhodopsin, which decays thermally to an intermediate containing an *all-trans*-retinal chromophore, known as metarhodopsin (Kitagawa & Tsuda, 1980; Doukas et al., 1980). Resonance Raman studies have shown that the retinal chromophore in octopus rhodopsin and octopus bathorhodopsin, like their bovine counterparts, is attached to the lysine via a protonated Schiff base linkage (Pande et al., 1987;

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Oseroff & Callender, 1974; Aton et al., 1980). Calorimetric studies have shown that almost the same amount of photon energy is converted to chemical energy during the rhodopsin-to-bathorhodopsin transition for the two systems (Cooper, 1979; Cooper et al., 1986; Schick et al., 1987). And finally, comparison of the amino acid sequences for octopus and bovine rhodopsin shows significant homology between the two primary structures (Ovchinnikov et al., 1988).

Despite these broad similarities, however, there are some interesting differences in the photochemical behavior of the vertebrate and invertebrate pigments which are displayed in the differences between the octopus and bovine pigments. In the invertebrate systems, metarhodopsin is stable under physiological conditions, while vertebrate metarhodopsin is not (Tsuda, 1979, 1982a). This leads to the eventual dissociation of the chromophore in the vertebrate rhodopsin. Furthermore, invertebrate metarhodopsin reverts preferentially to rhodopsin upon illumination (Hamdorf et al., 1972; Suzuki et al., 1976), while vertebrate metarhodopsin does not. Also, unlike bovine rhodopsin, illumination of octopus rhodopsin at liquid helium temperatures produces large amounts of a blue-shifted photointermediate, hypsorhodopsin, besides the usual bathorhodopsin and isorhodopsin species (Tsuda et al., 1980), and illumination of octopus rhodopsin with violet light results in the formation of a 13-cis pigment (Ohtsu & Kito, 1985).

We report here infrared studies of octopus rhodopsin and its low-temperature photointermediates, bathorhodopsin and isorhodopsin. The main objective of this study is to make comparisons between octopus rhodopsin, an invertebrate system, and bovine rhodopsin, a vertebrate system. Such comparisons should provide information not only on the nature of the differences exhibited by the two systems but also on which aspects of the visual pigment photochemistry are pertinent in developing more detailed models concerning general mechanisms of light–energy transduction for both vertebrate and invertebrate visual systems.

Our infrared spectra provide evidence that octopus bathorhodopsin, like bovine bathorhodopsin, contains an all-trans isomer of retinal. This result, taken together with chemical extraction results which show that octopus rhodopsin contains 11-*cis*-retinal, provides compelling evidence that the primary effect of light on octopus rhodopsin is 11-cis/11-trans isomerization of the retinal chromophore. However, we find dramatic differences in the two systems as well. Namely, both the rhodopsins and the isorhodopsins differ significantly in their fingerprint region (1100–1400 cm^{-1}), and the two bathorhodopsins show marked differences in lines attributable to the hydrogen out-of-plane (HOOP)[1] modes of the retinal chromophore. In addition, both similarities and differences in lines attributable to changes in the opsin are found. Thus, it seems clear that while the general mechanism of pigment photochemistry for the two systems is similar, there may be important differences in specific protein–chromophore interactions between the octopus and bovine systems.

Materials and Methods

Octopus (Mizudako, *Paroctopus defleini*) microvillar membranes were prepared as previously described (Tsuda, 1979). These membranes were diluted into water and then dried under a vacuum onto an infrared and visibly transparent window (SrF_2, Optovac Inc.). The film was then mounted in a specially designed humidity cell [see Bagley (1987)] that was sealed via a second infrared-transparent, but visibly opaque, window (antireflection-coated Ge, Exotic Materials Inc.). The absorbance of the films was approximately 0.1 OD at 500 nm.

[1] Abbreviations: HOOP, hydrogen out of plane; R, rhodopsin; B, bathorhodopsin; I, isorhodopsin; bR, bacteriorhodopsin; HPLC, high-pressure liquid chromotography.

Table I: Percentages of Octopus Rhodopsin, Octopus Isorhodopsin, and Octopus Bathorhodopsin in Photostationary States Created by Illumination with Light of Various Wavelengths[a]

λ (nm)	% rhodopsin	% isorhodopsin	% bathorhodopsin
460[b]	22.2	19.6	58.4
500[b]	24.7	31.7	43.6
560[c]	12.0	88.0	0.0
600[b]	60.3	32.3	7.4

[a] The photostationary states were created by illuminating octopus microvilli fragments, cooled to 85 K, with light from a projector equipped with interference filters. [b] Percentages found by using chemical extraction as described in text. [c] Percentages found by using the method of Yoshizawa and Wald (1964).

The sample holder containing the octopus microvilli film was mounted in a helium refrigerator equipped with NaCl windows. A Mattson Sirius Fourier transform infrared spectrometer equipped with an HgCdTe detector was used to record the spectra. To filter out incident visible radiation from the infrared source, and yet illuminate the sample with visible light during the course of the experiment, the sample holder was mounted such that the Ge window was between the octopus microvilli film and the infrared source. The sample was illuminated through the SrF_2 window.

The sample was cooled to 85 K and an infrared transmission spectrum collected at 2-cm^{-1} resolution over the spectral region from 2000 to 700 cm^{-1}. To eliminate base-line drift, 512 scans were collected through the sample, 512 scans were collected from a background compartment containing no sample, and the ratio of the sample to background was calculated. This process was repeated until four sucessive transmission spectra had been collected. The four spectra were then averaged, resulting in a transmission spectrum with an adequate signal-to-noise ratio.

Four illumination wavelengths were used to create photostationary states (460, 500, 560, and 600 nm). The relative percentages of bathorhodopsin (B), isorhodopsin (I), and rhodopsin (R) for the various photostationary states formed are shown in Table I. For illumination at 460, 500, and 600 nm the relative percentages for B, I, and R were determined as follows: following illumination at 85 K, the octopus microvilli were warmed to room temperature in the dark, resuspended in hydroxylamine (2.0 M), and extracted as described previously (Tsuda, 1982b). Relative percentages of the various isomers present were then determined by using high-pressure liquid chromotography (HPLC). The percentages of 11-*cis*-, 9-*cis*-, and *all-trans*-retinals found in the extract were assumed to be equal to the percentages of R, I, and B in the photostationary states formed at 85 K, respectively. We note that the percentage of 11-*cis*-retinal in the unilluminated octopus rhodopsin was found to be equal to or greater than 95%, thus confirming that octopus rhodopsin contains an 11-cis isomer of retinal. Furthermore, the percentages of B, I, and R determined by fitting the 460- and 500-nm photostationary-state spectra with a weighted sum of the visible spectra of B, I, and R (Tsuda, unpublished results) give quite similar results to those found with our extraction/HPLC determination technique, thus confirming the assumption inherent to the extraction/HPLC tecnnique, namely, that octopus rhodopsin contains 11-*cis*-retinal, octopus isorhodopsin contains 9-*cis*-retinal, and octopus bathorhodopsin decays to *all-trans*-retinal. The relative percentages of B, I, and R in the photostationary state formed with 560-nm illu-

mination were determined by the visible absorption vs bleaching time method of Yoshizawa and Wald (1964).[2]

As can be seen from Table I, illumination with 560-nm light creates a photostationary state that contains a large percentage of isorhodopsin and no bathorhodopsin. Therefore, an infrared difference spectrum between isorhodopsin and rhodopsin can be obtained directly. Likewise, inspection of Table I shows that the difference spectrum formed by subtraction of the infrared spectrum for the photostationary state formed with illumination of 500 nm and the photostationary state formed by illumination at 600 nm yields a difference spectrum equivalent to a B/R difference spectrum with less than a 2% contribution from isorhodopsin. Unfortunately, one cannot obtain a difference spectrum between bathorhodopsin and isorhodopsin directly. However, by subtracting photostationary-state difference spectra with appropriate weighting factors, calculated from the percentages given in Table I, one can obtain the difference spectrum between B and I (B/I). As a check on the reliability of this spectrum we performed subtractions using different combinations of the original photostationary-state difference spectra, calculated to yield B/I difference spectra, and obtained very similar spectra.

The infrared vibrational frequencies given in this paper are those that occur in the difference spectra. No corrections have been made for distortions in the shape and location of the peaks that occur when difference spectra are calculated, nor were the spectra smoothed. The octopus B/R infrared difference spectrum is shown in Figure 1Aa, the octopus B/I difference spectrum is shown in Figure 1Ab, and the octopus I/R difference spectrum is shown in Figure 1Ac. For comparison purposes, the B/R, B/I, and I/R infrared difference spectra for bovine rod outer segments are shown in panels Ba, Bb, and Bc of Figure 1, respectively. The bovine infrared difference spectra were formed as described in Bagley et al. (1985). Throughout the text, figure numbers followed by a plus sign designate the positive or upward-going lines in the spectrum while figure numbers suffixed by a minus sign designate the negative or downward-going lines for the figure. For example, Figure 1Aa+ refers to the upward-going lines in Figure 1Aa and Figure 1Aa− refers to the downward-going lines.

Results and Discussion

Comparison of the Infrared Difference Spectra of Octopus Rhodopsin and Bovine Rhodopsin. The C=C stretching frequencies of retinal are expected in the spectral region between 1500 and 1610 cm^{-1}. Two lines for octopus rhodopsin, located at 1562 and 1554 cm^{-1}, are found in this region of the infrared spectra. (See Figure 1Aa−,c−. We note that the 1562-cm^{-1} R line is not detected in Figure 1Ac−; this apparent contradiction is resolved by assuming that the 1562-cm^{-1} R line in Figure 1Ac− is canceled by a more intense line at 1565 cm^{-1} in I.) In contrast, the resonance Raman spectrum of octopus rhodopsin (Pande et al., 1987) displays a single line at 1548 cm^{-1}. This difference between the infrared difference spectra and the resonance Raman spectrum for octopus rhodopsin is similar to the results found in bovine rhodopsin, i.e., the infrared spectra of bovine rhodopsin has two lines at 1556 and 1547 cm^{-1}. [See Figure 1Ba−,c−. We note that the observation that a single line at ca. 1558 cm^{-1} is seen in Figure 1Ba− and no line is seen in Figure 1Bc− is not easily reconciled to the statement that R has two lines at ca. 1547 and 1556 cm^{-1}. See Bagley et al. (1985) for discussion of why there is only a single line at 1558 cm^{-1} in Figure 1Ba− and no line in Figure 1Bc−.] The resonance Raman spectrum for bovine rhodopsin has a single line at 1545 cm^{-1} (Callender et al., 1976; Mathies et al., 1977). The infrared line at 1554 cm^{-1} in octopus rhodopsin displays an inverse relationship between the visible absorption maximum, λ_{max} = 485 nm, and the main Raman and infrared-active ethylenic stretching frequency observed for retinal and retinal-based pigments (Rimai et al., 1973; Aton et al., 1977), suggesting that the 1554-cm^{-1} line in the infrared of octopus rhodopsin arises from an ethylenic vibrational mode of the retinal. While the nature of the additional line in both rhodopsins is at present unknown, we tentatively attribute them to infrared-active, Raman-inactive ethylenic modes of the retinal.

The spectral region between 1300 and 1100 cm^{-1} is commonly referred to as the fingerprint region for retinals since in free retinals and their protonated Schiff bases this region is characteristic of the isomeric state of the retinal chain. In bovine rhodopsin, the pattern of lines in this region remains relatively unperturbed from that found in free 11-*cis*-retinal or its protonated Schiff base. It is therefore quite dramatic that octopus rhodopsin which extracts as 11-*cis*-retinal (see Materials and Methods) exhibits a very different pattern of lines in this region of the IR spectrum: the infrared spectrum for octopus rhodopsin exhibits a single intense line at 1226 cm^{-1} (see Figure 1Aa−,c−) in contrast to the quadruplet of lines (at 1251, 1238, 1216, and 1191 cm^{-1}) that is found in the infrared spectra of bovine rhodopsin (compare Figure 1Aa−,c−, to Figure 1Ba−,c−). These differences in the fingerprint region of the infrared spectra between octopus rhodopsin and bovine rhodopsin are in good agreement with differences detected in the resonance Raman spectra of Pande et al. (1987) and thus confirm that these lines arise from vibrations of the retinal rather than changes in the vibrations of the opsin. We suggest that the differences in the fingerprint region between octopus rhodopsin and bovine rhodopsin, each of which extracts as 11-*cis*-retinal, may reflect differences between the two species in their protein–chromophore interactions. We note that such differences in the protein–chromophore interactions between octopus and bovine rhodopsin are not unexpected [see, e.g., Koutalos et al. (1989)].

In the 800–1000-cm^{-1} region of the infrared spectrum, octopus rhodopsin has a single line at 973 cm^{-1} (Figure 1Aa−,c−), in close agreement with a line at 970 cm^{-1} in the infrared spectra of bovine rhodopsin (Figure 1Ba−,c−). The 970-cm^{-1} line in bovine rhodopsin has been assigned to a coupled 11-H/12-H hydrogen out-of-plane bending mode of the retinal (Eyring et al., 1982).

Comparison of the Infrared Spectra of Octopus and Bovine Bathorhodopsin. In the infrared spectral region between 1500 and 1610 cm^{-1}, octopus bathorhodopsin exhibits two lines at 1584 and 1532 cm^{-1} (see Figure 1Aa+,b+). Both of these lines have their counterparts in the IR spectra of bovine bathorhodopsin, which has lines at 1579 and 1536 cm^{-1} (see Figure 1Ba+,b+). For bovine bathorhodopsin both lines have been attributed to ethylenic modes of the retinal chromophore (Bagley et al., 1985).

The fingerprint region for octopus bathorhodopsin closely resembles that of bovine bathorhodopsin. A tentative as-

[2] We note that the HPLC determination technique discussed above did not give reproducible values from experiment to experiment for illuminations at 560 nm, nor were these values consistent with the values obtained according to the method of Yoshizawa and Wald. We are unable to explain this observation. However, the self-consistency of the infrared difference spectra formed by using various combinations of the illumination wavelengths strongly supports the assumption that the percentages of B, I, and R in the photostationary state formed at 85 K with 560-nm illumination are given accurately by Yoshizawa and Wald's method.

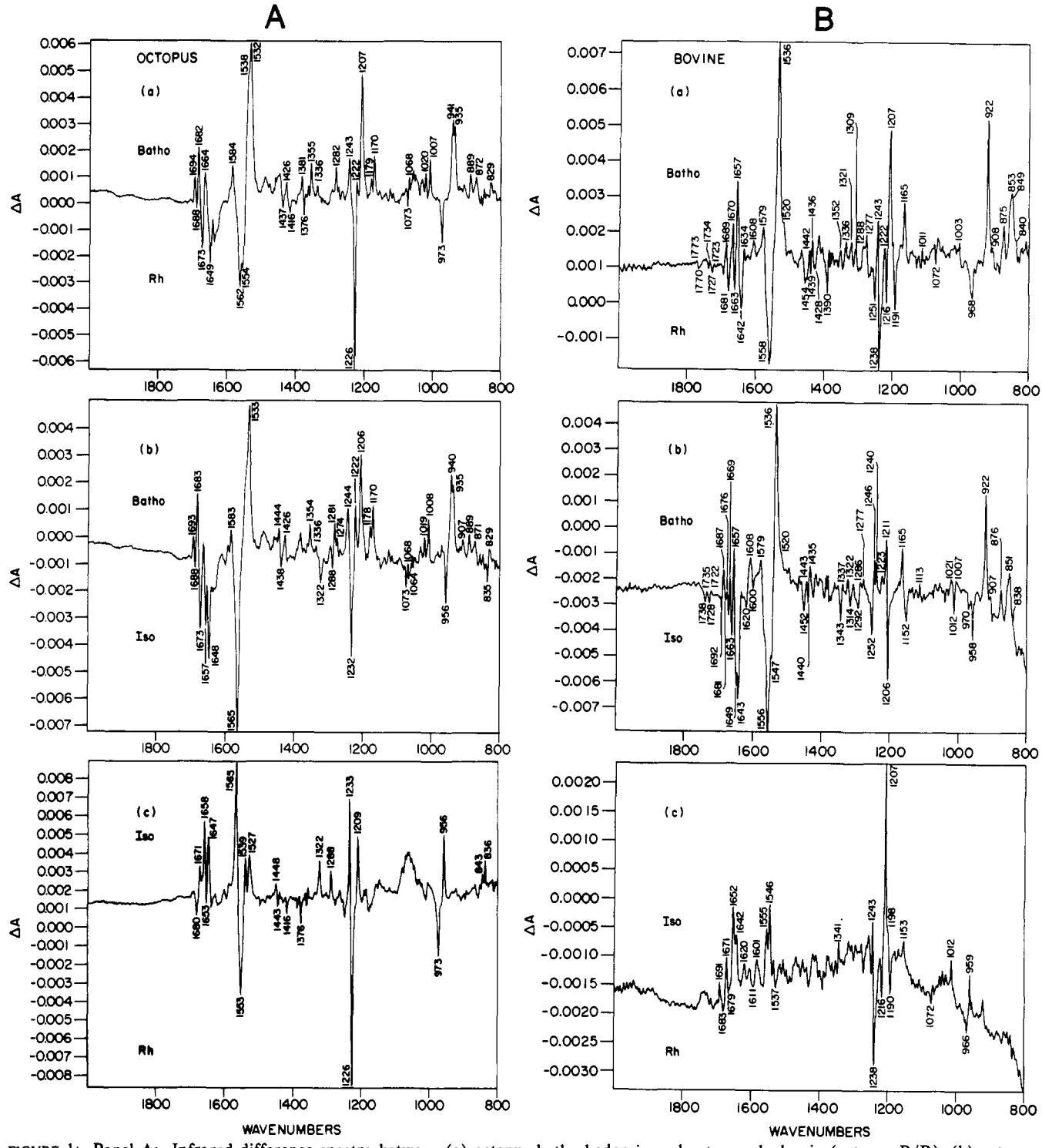

FIGURE 1: Panel A: Infrared difference spectra between (a) octopus bathorhodopsin and octopus rhodopsin (octopus B/R), (b) octopus bathorhodopsin and octopus isorhodopsin (octopus B/I), and (c) octopus isorhodopsin and octopus rhodopsin (octopus I/R). The infrared difference spectra were calculated by subtracting photostationary-state difference spectra with appropriate weighting factors (calculated from the concentrations given in Table I). The photointermediates were formed at 85 K. Spectral resolution is 2 cm^{-1}. Panel B: Infrared difference spectra between (a) bovine bathorhodopsin and bovine rhodopsin (bovine B/R), (b) bovine bathorhodopsin and bovine isorhodopsin (bovine B/I), and (c) bovine isorhodopsin and bovine rhodopsin (bovine I/R). The infrared difference spectra were formed as described in Bagley et al. (1985). The photointermediates were formed at 70 K. Spectral resolution is 2 cm^{-1}. Note: Due to the nature of the subtractions used to create the difference spectra, the ΔA scales in these spectra are somewhat arbitrary.

signment of the bovine bathorhodopsin lines has been given by Palings et al. (1987). We detect lines for octopus bathorhodopsin at 1354, 1336, 1281, 1244, 1206, and 1170 cm^{-1} (Figure 1Aa+,b+), while bovine bathorhodopsin has lines at 1352, 1336, 1282, 1244, 1222, 1207, and 1165 cm^{-1} (Figure 1Ba+,b+). The line at ca. 1207 cm^{-1} in both pigments is the most intense feature in this spectral region. The only major difference between the two pigments in this region of the spectrum is a new line in the octopus bathorhodopsin spectra at 1179 cm^{-1} which is not seen in the bovine bathorhodopsin spectra.

The existence of a line in octopus bathorhodopsin at 1170 cm^{-1} is of particular interest. A line is found at ca. 1170 cm^{-1} in the resonance Raman and infrared spectra of *all-trans*-retinal (Callender et al., 1976; Cookingham et al., 1978; Curry et al., 1982) and its protonated Schiff base (Mathies et al.,

1977; Braiman & Mathies, 1980; Smith et al., 1985; Crouteau, 1986) which is characteristic of the all-trans isomer of retinal. Bovine bathorhodopsin and the light-adapted form of bacteriorhodopsin, two pigments that contain all-trans isomers of retinal, exhibit lines in the infrared spectra at 1165 cm^{-1} (Bagley et al., 1985) and 1169 cm^{-1} (Bagley et al., 1982), respectively. The existence of a line in octopus bathorhodopsin at 1170 cm^{-1} therefore provides further evidence that octopus bathorhodopsin, like bovine bathorhodopsin, contains an all-trans-like isomer of retinal.

Dramatic differences between the infrared spectra for bovine and octopus bathorhodopsins are found in the spectral region between 800 and 1000 cm^{-1}. Bovine bathorhodopsin has intense lines in this region of the spectrum at 922, 875, 853, and 849 cm^{-1}, with additional weak lines appearing at 908 and 840 cm^{-1} (Figure 1Ba+,b+). Octopus bathorhodopsin exhibits a strong doublet at 941 and 935 cm^{-1} with three weaker lines appearing at 889, 872, and 829 cm^{-1} (see Figure 1Aa+,b+). The infrared spectra for octopus bathorhodopsin are in general agreement with the resonance Raman results of Pande et al. (1987).[3]

Differences in this region of the vibrational spectrum are particularly interesting since it has been suggested that the 922-cm^{-1} line in bovine bathorhodopsin arises from an isolated 11-H hydrogen out-of-plane mode of a tortionally distorted all-trans chromophore that is perturbed by a negatively charged protein residue near the C11=C12 moiety of the retinal chromophore (Eyring et al., 1982; Bagley et al., 1985). Differences in the octopus bathorhodopsin and bovine bathorhodopsin lines in this region are therefore suggestive of differences in distortions from planarity of the *all-trans*-retinal chromophores for the two pigments and/or in the interactions between the protein and chromophore for these two batho products.

Comparison of the Infrared Difference Spectra for Octopus Isorhodopsin and Bovine Isorhodopsin. A single ethylenic line appears at 1565 cm^{-1} in the infrared spectra of octopus isorhodopsin (λ_{max} = 460 nm) (Figure 1Ab–,c+). This ethylenic is in good agreement with the inverse relationship displayed between the main ethylenic and the visible absorption maximum for retinals and retinal-based proteins (Aton et al., 1977). In contrast, bovine isorhodopsin has two IR-active modes attributable to ethylenic modes of the retinal: a line at 1556 cm^{-1} that displays the above-mentioned inverse relationship to its visible absorption maximum, and an infrared-active, resonance Raman inactive line at 1547 cm^{-1}.

The most intense line in the fingerprint region of the infrared spectra of octopus isorhodopsin is located at 1233 cm^{-1}, with smaller lines at 1322, 1288, and 1209 cm^{-1} (Figure 1Ab–,c+). In marked contrast, bovine isorhodopsin has lines at 1341, 1252, 1206, and 1153 cm^{-1} (Figure 1Bb–,c+). In addition, unlike bovine isorhodopsin, octopus isorhodopsin, which also extracts as 9-*cis*-retinal (see Materials and Methods), displays a very different pattern of lines in the fingerprint region than those found for 9-*cis*-retinal and its protonated Schiff base. An understanding of these differences awaits assignment of these lines to vibrational modes for octopus isorhodopsin.

Finally, in the 800–1000-cm^{-1} region of the infrared spectrum, octopus isorhodopsin has a single line at 956 cm^{-1} (Figure 1Ab–,c+), in close agreement with a line at 959 cm^{-1}, in bovine isorhodopsin, that has been assigned to a coupled 7-H/8-H hydrogen out-of-plane bending mode of retinal (Eyring et al., 1982; Bagley et al., 1985).

Comparison of Lines Attributable to Protein Changes in the Infrared Difference Spectra for the Octopus and Bovine Rhodopsins and Their Intermediates. In the B/R difference spectrum for bovine rhodopsin, highly reproducible lines at 1773 cm^{-1} for bathorhodopsin and at 1770 cm^{-1} for rhodopsin were detected, which were tentatively attributed to changes associated with –COOH groups of aspartic or glutamic acid residues of the apoprotein on the basis of their shift upon medium deuteration to 1762 and 1756 cm^{-1}, respectively (Siebert et al., 1983; Bagley et al., 1985). Additional lines at 1735 and 1722 cm^{-1} for bovine bathorhodopsin and at 1741 and 1728 cm^{-1} for bovine rhodopsin were found to be insensitive to medium deuteration and were thus proposed to arise either from changes in nonexchangeable –COOH residues of glutamic or aspartic acid in the opsin or from changes in ester groups of the rod outer segment lipids during the rhodopsin-to-bathorhodopsin transition. In contrast, our octopus B/R, B/I, and I/R infrared difference spectra show no discernible lines between 1700 and 1800 cm^{-1}.

To try to understand some of these differences, we compared the primary sequences for bovine and octopus rhodopsins. The unusually high frequencies of 1773 and 1770 cm^{-1} for these –COOH bands in bovine B and R, respectively, indicate that the carbonyl of the carboxyl group is not hydrogen-bonded and likely arises from a protonated glutamic or aspartic acid in a hydrophobic environment (Bellamy, 1957). Therefore, we compare only those parts of the sequences that are predicted to lie in the membrane bilayer of the two pigments.

Ovchinnikov et al. (1988) have recently reported the complete primary sequence for octopus rhodopsin. They note that the only carboxylic amino acid predicted to lie within the membrane bilayer of octopus rhodopsin is Asp-81. Furthermore, this aspartic acid is found to be conserved in all known visual pigments, thus leading to the suggestion that it may provide the counterion for the protonated retinal–lysine Schiff base (Applebury & Hargrave, 1986). The bovine rhodopsin sequence places two glutamic acids within the membrane bilayer that are not found in octopus rhodopsin (Glu-122 and Glu-134 in helix III). On the basis of these observations a reasonable interpretation of the infrared spectra is that the –COOH bands we detect in the infrared spectra for bovine rhodopsin arise from differences in either environment or protonation state which Glu-122 and/or Glu-134 experience during the rhodopsin-to-bathorhodopsin transition.

In the bovine B/R and B/I infrared difference spectra, a line[4] is detected for B at ca. 1520 cm^{-1}. The position of this line is insensitive to isotopic substitution on the retinal [data not shown; see Bagley (1987)] and is not detected in resonance Raman experiments, thus suggesting that it arises from a change in a normal mode of the opsin during the rhodopsin-to-bathorhodopsin and isorhodopsin-to-bathorhodopsin tran-

[3] The resonance Raman and infrared spectra for bathorhodopsin are in general agreement with the exception that the resonance Raman spectra show two lines for octopus bathorhodopsin at 957 and 971 cm^{-1} that are not detected in the infrared spectra. While these additional lines in the resonance Raman spectra may represent Raman-active, infrared-inactive lines in the octopus bathorhodopsin spectra, we feel it is possible that these lines might also arise from small contributions from the rhodopsin and isorhodopsin intermediates, respectively.

[4] The B 1520-cm^{-1} line appears as a shoulder on the side of the B ethylenic ca. 1532-cm^{-1} line in the infrared spectra that are presented here. Stronger evidence of its existence is provided by its high reproducibility from experiment to experiment and the fact that it appears fully resolved in the B/R and B/I infrared difference spectra for bovine rod outer segments suspended in deuterated medium and in the B/R and B/I infrared difference spectra of rhodopsins containing isotopically labeled retinals, where the ethylenic in B shifts to higher frequencies (Bagley, 1987).

sitions. The position of this line is also insensitive to medium deuteration [see Bagley et al. (1985)].

We do not resolve a B line at ca. 1520 cm^{-1} (nor any hint of a shoulder) in the B/R and B/I infrared difference spectra for octopus rhodopsin. However, given the lower frequency of the B ethylenic (1532 cm^{-1}) for octopus compared to that of the bovine B ethylenic (1536 cm^{-1}), we cannot rule out the possibility that the line is merely hidden under the more intense B ethylenic in the octopus B/R difference spectra.

The above observations are interesting because in the infrared difference spectra between bacteriorhodopsin and its primary photoproduct, K, a line for K is detected at 1518 cm^{-1} (Dollinger et al., 1986; Rothschild et al., 1986) and has been assigned to a normal mode of a protonated tyrosine. Thus we suggest that in the bovine B/R and B/I infrared difference spectra, the bathorhodopsin line at 1520 cm^{-1} arises from changes in either the environment or the state of protonation of a tyrosine during the bovine rhodopsin-to-bathorhodopsin and isorhodopsin-to-bathorhodopsin transitions. The apparent lack of a similar line in the octopus rhodopsin difference spectra suggests that this feature arises from a tyrosine residue that is not conserved in the amino acid sequences for the two pigments. Comparison of the sequences for the two pigments shows that the tyrosine nearest to the active site in the bovine pigment is replaced by a histidine in the octopus pigment, thus suggesting that the changes detected in a tyrosine during the bovine rhodopsin photosequence arise from Tyr-301. Furthermore, the apparent lack of this line in the octopus spectra suggests that the changes in tyrosines which give rise to this feature in the infrared spectra are not essential to light–energy transduction.

Similar to the bovine B/R, I/R, and B/I difference spectra, the octopus B/R, I/R, and B/I difference spectra show many lines in the 1620–1700-cm^{-1} region of the infrared spectrum. As in the B/I, I/R, and B/R infrared difference spectra for the bovine system, we attribute these lines in the octopus difference spectra to carbonyls of the protein backbone within the chromophore pocket that experience small changes in environment in the transitions between B, I, and R. The argument is as follows. The only vibrational mode of the retinal expected in this region of the infrared spectrum is the C=NH stretching frequency of the retinal protonated Schiff base linkage. This expectation is consistent with recent resonance Raman results that detect a single line at 1657 cm^{-1} in both octopus bathorhodopsin and octopus rhodopsin. In each case this line was assigned to the C=NH stretch of a protonated Schiff base linkage on the basis of its shift to lower frequencies upon medium deuteration (Kitagawa & Tsuda, 1980; Pande et al., 1987). The absence of a line for either bathorhodopsin or rhodopsin near 1657 cm^{-1} in the octopus B/R infrared difference spectrum is consistent with the near degeneracy found for the C=NH stretch for B and R in the resonance Raman spectra. Furthermore, since the C=NH stretch of retinal is the only retinal vibrational mode found in this region of the spectrum, all other infrared lines between 1620 and 1700 cm^{-1} in the octopus B/R IR difference spectrum must arise from vibrational modes of the protein that change during the rhodopsin-to-bathorhodopsin transition. Since amide I has a broad absorption, ca. 1660 cm^{-1}, we propose that lines detected in this region of the infrared spectrum result from changes in the environment of amide bonds located near the chromophore pocket. More specifically, it seems reasonable to expect that the cis/trans isomerization of the retinal during the rhodopsin-to-bathorhodopsin transition would result in small changes in the environment of the amides near the lysine to which the retinal is attached. We attribute lines between 1620 and 1700 cm^{-1} in the octopus B/I and I/R difference spectra, with the possible exception of the 1657-cm^{-1} line for octopus isorhodopsin,[5] to changes in amide I as well, using similar arguments.

Conclusions

The results of this study have corroborated many of the conclusions reached through recent resonance Raman studies of octopus rhodopsin and its bathorhodopsin photointermediate (Pande et al., 1987). Our infrared spectra of octopus bathorhodopsin show significant similarities to the infrared and resonance Raman spectra of bovine bathorhodospin and *all-trans*-retinal. In particular, the infrared spectra for octopus bathorhodopsin show the existence of a line at ca. 1170 cm^{-1} which is characteristic of an all-trans isomer of retinal. These results taken together provide compelling evidence that the retinal in octopus bathorhodopsin is all-trans. Furthermore, since chemical extraction studies show that octopus rhodopsin extracts as 11-*cis*-retinal, we conclude that the primary effect of light absorption by octopus rhodopsin, like that of bovine rhodopsin, is 11-cis/11-trans isomerization of the retinal chromophore.

The infrared difference spectra show that there are dramatic differences between the structure-sensitive fingerprint lines in octopus rhodopsin and bovine rhodopsin, in agreement with resonance Raman results (Pande et al., 1987). Our infrared spectra show that significant differences are also found in the fingerprint regions for the isorhodopsins of the two systems. Since octopus rhodopsin and octopus isorhodopsin contain 11-cis and 9-cis chromophores, respectively, these data suggest that the specific protein–chromophore interactions in the two systems may be quite different. Such differences are not unexpected. For example, it has been proposed that charged amino acid residues strategically located near the chromophore are responsible for color regulation in the visual pigments [see, e.g., Honig and Ebrey (1982)]. If this is the case, the differences in the fingerprints of the two pigments may reflect differences in the charge placement for the two pigments which give rise to their differences in color [see, e.g., Koutalos et al. (1989)]. Alternatively, it may be that steric interactions within the protein cavities deform the 11-*cis*- and 9-*cis*-retinal chromophores differently in the bovine and octopus pigments. Birge et al. (1988) have recently suggested that the fingerprint lines, which are dependent on σ-dominated (single-bond) force constants and are highly delocalized in nature, should be relatively insensitive to charge placement around the retinal in the absence of covalent or hydrogen-bonding interactions. On the basis of this suggestion we think that it is likely that the differences between the fingerprint lines in octopus and bovine rhodopsins and similarly between the octopus and bovine isorhodopsins reflect differences in distortions of the chromophore for these pigments, rather than differences in charge placement around the chromophores. If this is true, then the observation that the fingerprint lines for bovine rhodopsin and bovine isorhodopsin resemble those of free 11-*cis*- and 9-*cis*-retinals, respectively, while the octopus

[5] Our preliminary unpublished results for octopus isorhodopsin in deuterated media suggest that octopus isorhodopsin also contains a protonated Schiff base linkage with a C=NH stretching frequency near 1657 cm^{-1}. Therefore, the 1657-cm^{-1} line in octopus isorhodopsin in the B/I and I/R difference spectra may arise from a more intense 1657-cm^{-1} C=NH line in isorhodopsin than is found in octopus rhodopsin or octopus bathorhodopsin. We attribute all other lines in this region of the B/I and I/R infrared difference spectra to changes in the opsin during transitions between bathorhodopsin, rhodopsin, and isorhodopsin.

pigments do not, suggests that bovine rhodopsin and bovine isorhodopsin accommodate relaxed chromophores within their binding site while octopus rhodopsin and octopus isorhodopsin contain chromophores that are in some way distorted by their binding pocket.

The recent resonance Raman studies of Barry et al. (1987) show that the positions and intensities of the HOOP modes in the bathorhodopsins of a variety of vertebrate visual pigments are very similar to those found in bovine bathorhodopsin. The significant differences we find in the HOOP modes for octopus bathorhodopsin are therefore quite unusual and apparently reflect some unique difference in the protein–chromophore interactions of the octopus pigment as compared to all the other pigments studied. We suggest that the differences in the HOOP modes of octopus bathorhodopsin probably do not reflect the differences in counterion location such as is commonly thought to be responsible for color regulation since, as Barry et al. (1987) point out, pigments with a wide range of λ_{max}'s have very similar HOOP lines. Furthermore, it seems unlikely that the differences in the HOOP modes in the various bathorhodopsins are related to some general energy storage mechanism in the visual pigments as suggested by Eyring et al. (1982). We find that the HOOP modes in octopus bathorhodopsin are dramatically different from those in bovine bathorhodopsin, and yet the amount of chemical energy that is stored upon light absorption in the octopus and bovine systems is very similar, namely, 32 and 35 kcal/mol, respectively (Cooper et al., 1986; Schick et al., 1987). We therefore conclude that, while it seems clear that the differences in the HOOP modes of the two pigments are related to differences in the protein–chromophore interactions in the two pigments, the relation of these differences to ideas concerning general mechanisms for energy storage and/or color regulation in visual pigments is not at all clear. Selective isotopic labeling of the chromophore and further normal mode calculations should prove useful in probing the nature of these differences.

Finally, a major strength of infrared difference spectroscopy is its ability to detect changes that single amino acids in the opsin experience during the various stages of the photosequence. In this paper we present evidence that tyrosines may be involved in the initial stages of light–energy transduction in the bovine system. In addition, our infrared spectra show lines at ca. 1760 cm^{-1} in the bovine B/R difference spectrum. The approximately 10-cm^{-1} shift in these lines upon medium deuteration (Bagley et al., 1985) and their anomolously high frequency indicate that these lines arise from some change in one or more protonated carboxylic amino acids that are located in a non-hydrogen-bonded, hydrophobic environment. The pattern of lines we detect is consistent with either a change in environment of a single carboxylic amino acid or simultaneous protonation of one carboxlyc amino acid along with deprotonation of a second. We note that the apparent lack of these lines in the octopus B/R spectra is consistent with the interpretation that the lines we detect at ca. 1760 cm^{-1} in the bovine B/R difference spectra arise from changes Glu-122 and/or Glu-134 experiences during the rhodopsin-to-bathorhodopsin transition, since these residues are the only carboxylic amino acids that are predicted to lie in the membrane bilayer and are not conserved in the octopus rhodopsin sequence (Ovchinnikov et al., 1988).

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Registry No. *all-trans*-Retinal, 116-31-4; glutamic acid, 56-86-0.

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NMR Studies of Abasic Sites in DNA Duplexes: Deoxyadenosine Stacks into the Helix Opposite Acyclic Lesions†

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ABSTRACT: Proton and phosphorus NMR studies are reported for two complementary nonanucleotide duplexes containing acyclic abasic sites. The first duplex, d(C-A-T-G-A-G-T-A-C)·d(G-T-A-C-*P*-C-A-T-G), contains an acyclic propanyl moiety, P, located opposite a deoxyadenosine at the center of the helix (designated AP_P 9-mer duplex). The second duplex, d(C-A-T-G-A-G-T-A-C)·d(G-T-A-C-*E*-C-A-T-G), contains a similarly located acyclic ethanyl moiety, E (designated AP_E 9-mer duplex). The ethanyl moiety is one carbon shorter than the natural carbon–phosphodiester backbone of a single nucleotide unit of DNA. The majority of the exchangeable and nonexchangeable base and sugar protons in both the AP_P 9-mer and AP_E 9-mer duplexes, including those at the abasic site, have been assigned by recording and analyzing two-dimensional phase-sensitive NOESY data sets in H_2O and D_2O solution between −5 and 5 °C. These spectroscopic observations establish that A5 inserts into the helix opposite the abasic site (P14 and E14) and stacks between the flanking G4·C15 and G6·C13 Watson–Crick base pairs in both the AP_P 9-mer and AP_E 9-mer duplexes. The helix is right-handed at and adjacent to the abasic site, and all glycosidic torsion angles are anti in both 9-mer duplexes. Proton NMR parameters for the AP_P 9-mer and AP_E 9-mer duplexes are similar to those reported previously for the AP_F 9-mer duplex (F = furan) in which a cyclic analogue of deoxyribose was embedded in an otherwise identical DNA sequence [Kalnik, M. W., Chang, C. N., Grollman, A. P., & Patel, D. J. (1988) *Biochemistry 27*, 924-931]. These proton NMR experiments demonstrate that the structures at abasic sites are very similar whether the five-membered ring is open or closed or whether the phosphodiester backbone is shortened by one carbon atom. Phosphorus spectra of the AP_P 9-mer and AP_E 9-mer duplexes (5 °C) indicate that the backbone conformation is similarly perturbed at three phosphodiester backbone torsion angles. These same torsion angles are also distorted in the AP_F 9-mer but assume a different conformation than those in the AP_P 9-mer and AP_E 9-mer duplexes.

Abasic sites in DNA are created by the loss of a purine or pyrimidine residue (Lindahl, 1982; Loeb & Preston, 1987; Weiss & Grossman, 1987). Such lesions, which arise spontaneously or through the action of *N*-glycosylases, lack primary coding information. Unless repaired prior to DNA replication, apurinic/apyrimidinic (AP) sites promote misincorporation of nucleotides and mutagenesis (Loeb & Kunkel, 1982; Loeb, 1985). The presence of AP sites in DNA also leads to strand scission via β-elimination (Weiss & Grossman, 1987).

In naturally occurring AP sites, a dynamic equilibrium exists between the cyclic hemiacetal (structure 1) and open-chain aldehyde (structure 2) forms of the 2-deoxyribose residue.

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